Original Research Article

STRUCTURAL ELUCIDATION AND ANTIMICROBIAL ANALYSIS OF BIOACTIVE COMPOUNDS FROM AMARANTHUS SPINOSUS

Abstract

Amaranthus spinosus is an herb which belongs to the Amaranthaceae family and used by ethnic people in the treatment of various infections and ailments. As a result of its medicinal properties, it is gaining the attention of many researchers.

This research work was carried out to isolate the compounds present in the leaves of *Amaranthus spinosus*, characterize, use obtained data to validate its bioactivity.

The crude extract was obtained using methanol in a Soxhlet extractor. The pure extracts were isolated from the crude extracts using column chromatography and further purification was done using Sephadex. The structures of the isolates were elucidated using nuclear magnetic Resonance Spectroscopy.

In the validation of the bioactivity of the plant, some pathogens were used, including *Escherichia coli, Bacillus species, and Pseudomonas. aeruginosa, Candida albicans and Aspergillus niger*.

The antimicrobial analysis carried out using two isolates from the plant extracts indicated the following zones of inhibition (mm) for the first isolate: 16.00±0.01, 16.00±0.01, 15.00± 0.08, 16.00±0.08, 17.00 ± 0.08. For the second isolate, the diameter zone of inhibition (mm) includes 6.00±0.08, 5.00± 0.10, 26.00± 0.10, 5.00±0.08, and 16.00±0.10. These results were compared with a standard antibiotic, ciprofloxacin,and standard antifungal, ketoconazole.

The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the isolated compounds were determined and compared with those of the standard drugs.

Keywords: Amaranthus spinosus, Antimicrobial, Pathogens, Nuclear Magnetic Resonance Spectroscopy.

Introduction

"The plant kingdom represents a rich storehouse of organic compounds, many of which have been used for medicinal purposes and could serve as lead for the development of novel agents having good efficacy in various pathological disorders in the coming years" (Dike et al., 2012).

Plants are medicinal and are of immense value to the health of individuals. Their medicinal importance lies in their bioactive components that produce physiological activities which are definite in the human body (Edeoga, 2005; Obazelu et al., 2021).

"The use of plant extracts and photoproducts is gaining attention due to their ability, cost-effectiveness, proven nature of specificity, biodegradability, low toxicity, and minimum residual toxicity in the ecosystem" (Maji et al., 2005). "Medicinal plants are considered a rich resource of ingredients that can be used in drug development and synthesis (Yudharaj et al., 2010). They have proved their sole role in coping with several deadly diseases including cancer and the diseases associated with viral onslaught viz Hepatitis, AID etc". (Refaz et al., 2017).

"The use of herbal medicine is on the increase globally" (Asimwe et al., 2014; Joshi and Joshi. 2000; Kamatenesi-Mugisha, 2005).

Ethnomedicine has been beneficial in the treatment of infections caused by bacterial pathogens, among its utmost benefits are its availability and ease of preparation, hence serving the ever-growing population of underdeveloped nations (Ijioma and Ajiwe, 2022).

"The use of various parts of plants in the prevention and treatment of many ailments is now experiencing positive awareness, especially amongst rural dwellers because of their availability, cheaper prices, effectiveness, and resistance to disease-causing organisms" (Arowosegbe et al., 2020).

"At present a substantial number of drugs are developed from plants that are active against a number of ailments and diseases conditions such as hypertension, pains, fever, cancer, diabetes, arthritis, gastrointestinal diseases and so on". (Olanipekun et al., 2021; Patel et al., 2010).

Amaranthus spinosus has a long history usage in traditional medicine against various ailments in the world (Barku et al., 2013). It is also used as green vegetables and cultivated in all over India (Chaudhary et al., 2012; Jhade et al., 2009).

"The juice of *Amaranthus spinosus* is used by the tribe of Kerala, India to prevent swelling around stomach while the leaves are boiled without salt and consumed for 2-3 days to cure jaundice. It is also used as anti-inflammatory, antimalaria, antibacterial, antimicrobial, antidiuretic, antiviral, and hepatic disorders". (Rai et al., 2014).

"The whole plant is used for the treatment of snake-bite, and thus acts as an antidote to snake venom. The root paste in an equal volume of honey is used to control vomiting". (Kirtikar and Basey, 2001).

"The plant is used in the treatment of abdominal pain, chicken pox, dysentery, dysuria, fever, hysteria, malaria, mania, tonsillitis, and vomiting" (Ishrat et al., 2011; Hussain et al., 2009).

"With the emerging world interest in adopting and studying traditional systems and exploiting their potential based on different health care systems, like evaluation of the rich heritage of traditional medicine is essential" (Srivastava et al., 2011). Hence, this work aimed at providing valid data which will prove the ethnomedicinal efficacy of *Amaranthus spinosus* and also add to the existing knowledge created by some researchers on the plant.

Materials And Methods

Collection of Plant Materials- Amaranthus spinosus plants were uprooted from uncultivated farmland in Awo-Omamma, Oru East Local Government Area, in Imo-state. The leaves were detached from the plants, washed, and air dried for 7 days under room temperature to avoid loss of active compounds.

The dried leaves were crushed into powder using a manual grinder. The powdered sample was then stored in an airtight container for further use.

Extraction of Plant Materials

200ml of methanol was taken into a 500ml round bottom flask. 500g of the powdered sample was placed in the Soxhlet extractor. The round bottom flask was attached to the extractor and the extraction lasted for about 3 hours. The extract

was collected and concentrated at a low temperature of about 40℃. The extract was cooled and stored in sample bottles for subsequent analysis.

Isolation of Compounds Using Column Chromatography

Column chromatography is a simple and most popular separation and purification technique. It consists of a stationary solid phase that adsorbs and separates the compounds passing through it with the help of a liquid mobile phase (Srivastiva, 2021).

A column was prepared using silica gel (60-120 mesh) and a solvent mixture of n-hexane and ethyl acetate in a ratio 90:10. The silica gel (adsorbent) was added as a fine dry powder in the column and the solvent mixture was allowed to flow freely through the column until equilibrium was achieved (Neha, 2021).

The extract was dissolved in a little quantity of the solvent mixture and was transferred to the top of the column which was covered with a small cotton wool to serve as a filter. Then the eluting solvent was added and the tap was opened to allow the eluting solvent to carry the extract through for proper isolation.

The eluent was collected using 50ml conical flasks. Isocratic elution technique was used hence further elution was done using the same solvent mixture. After the elution, the different isolated samples were further separated using Sephadex.

Further Purification Using Sephadex

25g of the Sephadex was dissolved in 150 ml of dichloromethane (Dcm) and methanol (50:50). The mixture was allowed to settle. The tap was opened to allow the solvent mixture to move down gradually until the level of the solvent mixture corresponds to that of the slurry. Each of the impure fractions from the column was dissolved in a small quantity of the solvent mixture (Dcm: methanol) and the mixture was introduced into a glass tube containing the Sephadex slurry. The sample was eluted using the solvent mixture. The eluent ($1cm^3$) was collected in intervals using test tubes. The test tubes and the contents were kept at room temperature for drying for about 2-3 days.

Thin Layer Chromatography of the Isolates

Each of the content of the test tubes was reconstituted using a mixture of Dcm: methanol (9:1). Aluminium foil TLC plates were given horizontal lines 1cm from the edge using a pencil. Marks and numbers were given on the horizontal lines depending on the number of fractions. The spotting was done using a micro

pipette. The TLC plates were developed in the solvent mixture of Dcm:methanol (9:1). After the development, the plates were viewed in a UV light. Similar fractions were pooled together and stored in weighed sample bottles with labels $ASLE_1$ and $ASLE_2$. After five days, the crystals were weighed and kept for further analysis.

Structural elucidation of the Isolated Compounds using NMR.

10mg of each of the isolates was dissolved in acetone and was analyzed for ^{1}H, ^{13}C, DEPT, COSY, HMBC, and HSQC in an NMR spectrometer (Varian-Vnmrs400) model.

Antimicrobial Analysis of the Isolated Compounds

Stock cultures of selected bacteria species and fungal cultures were obtained from the department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmacy, Nnamdi Azikiwe University, Agulu. The test organisms were prepared for the test.

Determination of the Diameter of the zone of inhibition using the Agar well diffusion method.

The agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts (Magaldi et al., 2004; Valgas., 2007). The agar well diffusion method used was described by Mounye et al., 2016).

The agar plate surfaces were inoculated by spreading 1ml each of the freshly prepared microbial inoculum over the entire agar surface. Then, 6 holes each with a diameter of 6mm were aseptically made on each plate. These were sufficiently spaced to prevent any inhibition zone from overlapping later on.

100μl of each extract (isolated compounds) and a control drug equivalent to a pre-determined concentration of 128%(w/v) were introduced into each well. The plates were kept for 30 minutes on the bench for the diffusion of the extracts to take place before incubation. The plates were incubated for 24 hours at 37℃. Later, zones of inhibition were inspected, measured, and recorded.

Determination of Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC).

Serial doubling dilutions of each extract were made, that is $\frac{1}{2}$, $\frac{1}{4}$,$\frac{1}{8}$,$\frac{1}{16}$, $\frac{1}{32}$, $\frac{1}{64}$ and $\frac{1}{128}$ by adding 2ml of each compound into the first bottle of 2ml of double-strength glucose indicator broth. From here, in a like manner, other dilutions in single-strength broth were made down to $\frac{1}{128}$ dilution. Tube 8 contained only media and culture, which can suppotrt growth of the organism. Tube 9 contained broth and extract to check the sterility of broth without organism inoculation. Tube 10 contained $\frac{1}{2}$ strength solvent cum broth to assess any deleterious effect of solvents on the microorganisms at lower dilutions.

Using a 3.0ml dropper sterile pipette, one drop of the appropriate inoculum, that is, the test organism was added respectively into each of the dilutions above. They were gently mixed and incubated at 37℃ for 48 hours.

To obtain the minimum inhibitory concentration (MIC) of the compounds, all those tubes showing no growth were tightly sub-cultured on fresh nutrient broth with a wire loop and incubated overnight at 37℃. The broth was used for the sub-culture in order to dilute out any carry over of residual extract.

Fresh nutrient agar was also used for the sub-culture to make sure there were no contaminating organisms.

The last tube showing growth was the minimum inhibitory concentration (MIC) of the compounds. Also, the minimum bacterial concentration (MBC) was in the last dilution tube showing no change in the broth.

Similarly, the above test was repeated using a standard antibiotic, Ciprofloxacin.

Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal concentration (MFC).

Seven McCartney bottles for each compound each containing 2ml $\frac{1}{2}$ strength solvent to correspond with the two fungal test organisms. The bottles were labelled 1-7 according to match with the compounds and fungal cultures. The dilutions

were also made as described above. The test was performed using punched agar diffusion method by Bryant ,1972.

The sabouraud dextrose agar plates were prepared, punched and inoculated as described above. The labelled wells were carefully filled with 2 drops of the different compound dilutions. From the wells, the compound dilutions diffused into the surrounding media and acted on the test organisms. The plates were kept for 30 minutes on the bench for proper diffusion. Thereafter, the plates were lifted very gently and placed with the base downwards in the incubator at 28℃- 30℃ for 48 hours. The plates were then examined for zones of inhibition. The control tubes 8,9 and 10 were first examined to ascertain that they behaved well and any abnormality was noted.

To obtain the minimum inhibitory concentration (MIC) of the compounds, all those tubes showing no growth were lightly sub-cultured on fresh sabouraud dextrose agar and incubated at 28℃- 30℃ for 48 hours . The last tube showing growth was the minimum inhibitory concentration (MIC) of the compound. Also, the minimum fungicidal concentration (MFC) is the last dilution tube showing no change in the broth. Similarly, the above test was done using a standard antifungal agent, Ketoconazole.

Results and Discussions

The result of the NMR analysis of the Isolated compounds.

The NMR analysis was carried out on the two compounds isolated from *Amaranthus spinosus* leaf extracts in the elucidation of their structures

ASLE$_1$

^{13}C NMR (400 MHz, acetone) δ 131.10 (d, *J* = 5.4 Hz), 128.72 (s), 64.99 (s), 37.12 (s), 33.34 (s), 31.78 (d, *J* = 5.7 Hz), 30.35 (d, *J* = 16.7 Hz), 29.81 – 28.30 (m), 25.21 (s), 24.74 (d, *J* = 6.9 Hz), 23.59 (s), 22.78 (d, *J* = 3.8 Hz), 22.44 (s), 20.96 (s), 20.59 (s), 18.96 (s), 18.49 (s), 13.50 (s), 13.13 (s), 11.65 (d, *J* = 19.0 Hz).

^{1}H NMR (400 MHz, acetone) δ 7.86 – 7.48 (m, 5H), 6.04 (dd, *J* = 34.1, 15.4 Hz, 1H), 5.20 (dd, *J* = 15.3, 8.8 Hz, 1H), 5.07 (dd, *J* = 15.1, 8.7 Hz, 1H), 4.49 – 4.35 (m, 1H), 4.35 – 4.14 (m, 5H), 4.07 (dd, *J* = 17.0, 10.0 Hz, 1H), 3.60 (s, 1H), 3.50 (dd, *J* = 13.0, 8.4 Hz, 1H), 3.33 – 3.22 (m, 1H), 3.06 – 2.96 (m, 1H), 2.84 (d, *J* = 18.2 Hz, 1H), 2.31 – 2.22 (m, 3H), 2.14 – 2.02 (m, 2H), 1.92 (d, *J* = 28.7 Hz, 2H), 1.85 (dd, *J* = 45.7, 29.7 Hz, 4H), 2.00 – 1.60 (m, 12H), 2.00 – 1.55 (m, 15H), 2.00 – -0.55 (m, 73H), 0.51 (d, *J* = 62.8 Hz, 4H), 0.51 (d, *J* = 62.8 Hz, 8H), 0.40 – 0.10 (m, 2H).

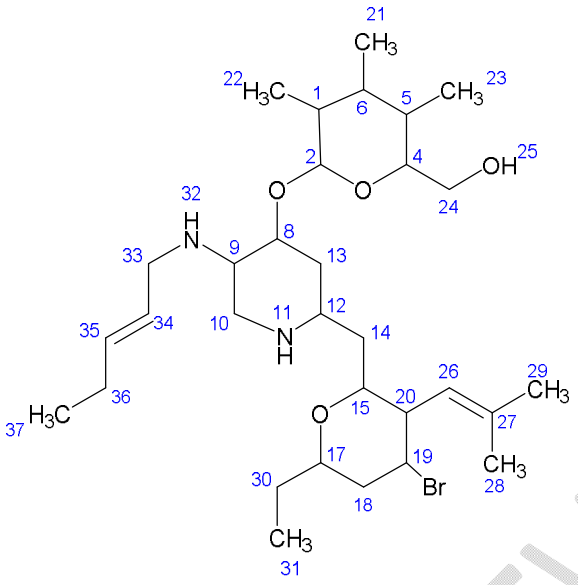

Figure 1: Structure of $ASLE_1$

Molecular Formula of $ASLE_1$: $C_{31}H_{55}BrN_2O_4$; Molecular weight of $ASLE_1$: 599.6834

IUPAC name: (E)-(6-((2-((4-bromo-6-ethyl-3-(2-methylprop-1-en-1-yl) tetrahydro-2H-pyran-2-yl)methyl)-5-(pent-2-en-1-ylamino)piperidin-4-yl)oxy)-3,4,5-trimethyltetrahydro-2H-pyran-2-yl)methanol

$ASLE_2$

^{13}C NMR (400 MHz, acetone) δ), 132.52 (d, *J* = 3.7 Hz), 131.09 (d, *J* = 5.5 Hz), 128.72 (d, *J* = 1.5 Hz), 67.39 (s), 64.97 (s), 38.74 (s), 30.34 (d, *J* = 16.8 Hz), 29.63 – 28.27 (m), 23.59 (s), 22.75 (s), 18.95 (s), 13.44 (s), 13.10 (s), 10.42 (s).

^{1}H NMR (400 MHz, acetone) δ 7.74 (dq, *J* = 7.4, 3.7 Hz, 19H), 7.70 – 7.56 (m, 22H), 6.08 (dd, *J* = 21.3, 7.8 Hz, 3H), 5.62 (ddd, *J* = 28.3, 12.6, 7.9 Hz, 8H), 5.29 – 5.15 (m, 6H), 5.08 (dd, *J* = 15.4, 8.6 Hz, 4H), 4.59 (d, *J* = 17.8 Hz, 3H), 4.49 – 4.35 (m, 5H), 4.32 – 4.14 (m, 44H), 4.04 (ddd, *J* = 15.4, 11.8, 5.8 Hz, 6H), 3.71 – 3.49 (m, 10H), 3.48 – 3.37 (m, 4H), 3.35 – 3.20 (m, 6H), 3.19 – 3.08 (m, 4H), 2.97 – 2.66 (m, 29H), 2.66 – 2.11 (m, 29H), 2.11 – 1.62 (m, 78H), 1.61 – 0.66 (m, 305H).

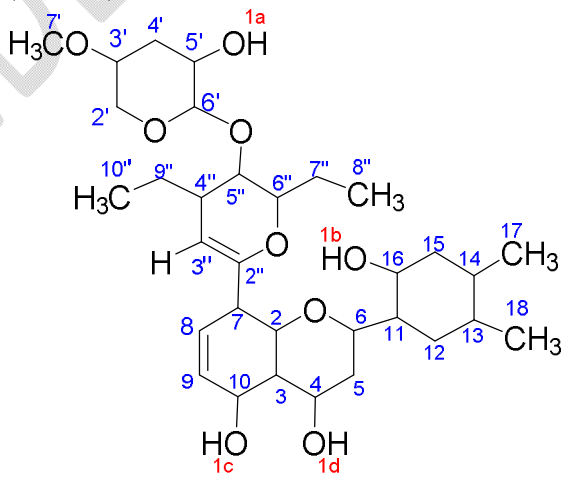

Figure 2: The structure of $ASLE_2$

Molecular formula of $ASLE_2$, $C_{32}H_{52}O_9$; Molecular weight of $ASLE_2$, 580.74988

IUPAC name: 8-(2,4-diethyl-5-hydroxy-3-((3-hydroxy-5-methoxytetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-6-yl)-2-(2-hydroxy-4,5-dimethylcyclohexyl)-3,4,4a,5,8,8a-hexahydro-2H-chromene-4,5-diol

Results of The Antimicrobial Activities

Table 1: Antimicrobial Activities of $ASLE_1$, $ASLE_2$, Ciprofloxacin and Ketoconazole

Extract code	Bacillus species	Escherichia coli	Pseudomonas aeruginosa	Candida albicans	Aspergillus niger
$ASLE_1$	16.00±0.01	16.00±0.01	15.00±0.08	16.00±0.08	17.00±0.08
$ASLE_2$	6.00±0.08	5.00±0.10	26.00±0.10	5.00 ±0.08	16.00±0.10
Ciprofloxacin	16.10±0.08	15.33±0.10	16.67±0.12	--	--
Ketoconazole	--	--	--	16.00±0.01	8.00±0.01

Key -- = no result

The antimicrobial screening results obtained from the Agar well diffusion method for Amaranthus spinosus methanolic extract showed a marked inhibitory effect against some of the test organisms.

For $ASLE_1$, the average diameter (mm) of the zone of inhibition was for Bacillus species (16.00± 0.01), E. coli (16.00±0.01), P. aeruginosa (15.00±0.08), C. albicans (16.00±0.08) and A. niger (17.00±0.08).

Similarly, for $ASLE_2$, the average diameter (mm) of the zone of inhibition for Bacillius species (6.00± 0.08), E. coli (5.00±0.10), P. aeruginosa (26.00±0.10), C. albicans (5.00±0.08) and A. niger (16.00±0.10).

The results above showed that the extract ASLE1 has high activity on the test organisms (bacteria and fungi). It was observed that the extract was more active

than the standard antibiotic, ciprofloxacin on E. coli and also more active than the antifungal agent , ketoconazole on A. niger. These results suggest that the extract will be very effective in the treatment of infections and diseases caused by these organisms. It can be employed in the manufacture of drugs caused by Escherichia coli, the commonest pathogen associated with patients with cystitis wounds, meningitis, diarrhea diseases like dysentery (Cheesbrough, 2002). Rabiu et al., 2020 reported the antimicrobial activity of Amaranthus spinosus. From the table, the activity of the extract on Bacillus species, P. aeruginosa, and C. albicans were almost the same as those of the standard drugs.

Similarly, the extract, ASLE2 showed very high activity on P. aeruginosa and A. niger which are higher than those of the standard drugs on these organisms while the standard drugs showed higher activity on B. species, E.coli, and C. albicans than the extract. Pseudomonas aeruginosa is an opportunistic pathogen affecting immunocompromised pathogens. It is known as the leading cause of morbidity and mortality in cystic fibrosis (CF) patients and as one of the leading causes of nosocomial infections (Morudali et al., 2017). These results showed that the leaf extract of Amaranthus spinosus can be used as both antibacterial and antifungal agent. Rai et al. (2014) have reported its use as an anti-inflammatory, antimalarial, antibacterial, antimicrobial, antidiuretic, anti-viral and hepatic disorder.

Table 2: Results of the MIC, MBC and MFC of $ASLE_1$, $ASLE_2$, Ciprofloxacin and Ketoconazole

Extract code	Activity	Bacillus species	Escherichia coli	Pseudomonas aeruginosa	Candida albicans	Aspergillus niger
$ASLE_1$	MIC	0.250	0.250	0.250	0.125	0.625
	MBC	0.50	0.50	0.50	--	--
	MFC	--	--	--	0.250	0.125
$ASLE_2$	MIC	0.50	0.50	0.625	1.00	0.625
	MBC	1.00	1.00	0.125	--	--
	MFC	--	--	--	0.50	0.125
C.FLOXACIN	MIC	0.250	0.250	0.250	--	--
	MBC	0.50	0.50	0.50	--	--

KETOCONAZOLE	MIC	--	--	--	0.125	0.125
	MFC	--	--	--	0.250	0.250

The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in tubes or microdilution wells as detected by the unaided eye (CLSI, 2012).

From the table, $ASLE_1$ gave the MIC's of 0.250, 0.250. 0.250, 0.125 and 0.625mg/ml towards Bacillus species, E. coli, P.aeruginosa, C.albicans and A. niger respectively.

Similarly, $ASLE_2$ gave the MIC's of 0.50, 0.50, 0.625, 1.00 and 0.625 mg/ml toward Bacillus species, E. coli, P. aeruginosa, c. albicans and A. niger respectively.

These extracts from Amaranthus spinosus had appreciable MICs compared with ciprofloxacin and ketoconazole.

MBC is defined as the lowest concentration of antibacterial agent that will prevent the growth of bacterium after the subculture on an antibacterial -free medium (Ijioma and Ajiwe, 2022).

MFC is defined as the lowest concentration of the antifungal agent that resulting in the death of 99.9% of the inoculum.

The MBC and MFC results of $ASLE_1$ showed that Bacillus species, E.coli, P. aeruginosa, C. albicans and A. niger were completely destroyed at the concentrations of 0.50,0.50, 0.50, 0.250 and 0.125mg/ml respectively.

Similarly, the MBC and MFC results of $ASLE_2$ for Bacillus species, E. coli, P. aeruginosa, C. albicans and A. niger were completely destroyed at the 1.00, 1.00, 0.125, 0.50 and 0.125mg/ml respectively.

These MBC and MFC results were comparable to those of the ciproflaxocin and ketoconazole respectively.

Ciprofloxacin completely exterminated Bacillus species, E. coli and P. aeruginosa at concentrations of 0.50mg/ml while Ketoconazole completely destroyed C. albicans and A. niger at 0.250mg/ml.

Based on the MIC, MBC and MFC, the extracts showed toxicity to all the test organisms at low concentrations indicating that the extracts at low concentrations are both antibacterial and antifungal agents. These findings also suggest that these two extracts could serve as drug in the pharmaceutical industries.

Conclusion

Amaranthus spinosus is no doubt a medicinal plant. The methanolic leaf extracts have both antibacterial and antifungal properties. Antimicrobial (antibacterial and antifungal) analyses showed that the extracts had broad spectrum antimicrobial properties and were comparable to antimicrobial agents (ciprofloxacin and ketoconazole) used in the assay. The extracts can be used in the treatment of infections caused by Pseudomonas aeruginosa and Aspergillus niger. This is in support of the claim of the ethnopeople in the medicinal use of the plants. These findings revealed that the potency of the extracts against the pathogens used could be as a result of the presence of naturally occurring bioactive phytochemicals present in the plant.

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